SECTION 2

CLINICAL INVESTIGATIONS

Chapter 5

Applications of stable isotopes in clinical pharmacology

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Abstract

This review aims to present an overview of the application of stable isotope technology in clinical pharmacology. For stable isotope technology in clinical pharmacology three main categories can be distinguished. Firstly, it is applied in the assessment of drug pharmacology to determine the pharmacokinetic profile or mode of action of a drug substance. Secondly, stable isotopes may be used for the assessment of drug products or drug delivery systems by determination of parameters such as the bioavailability or the release profile. Thirdly, patients may be assessed in relation to patient-specific drug treatment. The latter concept is often called personalized medicine. In this article the application of stable isotope technology in the aforementioned three areas is reviewed with a focus on the developments over the past 25 years. The applications are illustrated with examples from clinical studies in humans.

5.1 Introduction

The use of stable isotopes combined with mass spectrometry is widely accepted as a valuable research methodology. This is illustrated by the more than 33,000 hits obtained with the query "stable isotope" in the Scopus database (January 2011). Stable isotope technology is mainly applied in scientific areas as agriculture, biochemistry, earth and planetary sciences, environmental science and medicine. In pharmacology however, its use is relatively limited as evidenced by about 4,500 hits counted with the query "stable isotope" and "drug" (Scopus database, January 2011). The first paper describing the application of stable isotopes in this particular area dates from 1972 [1]. Knapp et al. investigated nortriptyline metabolism by a combination of a well-known experimental setup from radioactive tracer pharmacokinetic studies and stable isotope technology from metabolic studies. Since then, several groups have used stable isotope technology in their clinical pharmacological studies, especially those led by M. Eichelbaum and T.R. Browne. Their work has stimulated stable isotope technology to obtain a distinct place in clinical pharmacology in the 1980s. However, the most recent review paper on all applications of stable isotopes in (clinical) pharmacology is more than 20 years old [2]. Stable isotope applications in clinical pharmacology can be divided in three main categories. Firstly, it is applied in the assessment of drug pharmacology to determine the pharmacokinetic profile or mode of action of a drug substance. Secondly, stable isotopes may be used for the assessment of drug products or drug delivery systems by determination of parameters such as the bioavailability or the release profile. Thirdly, patients may be assessed in relation to patient-specific drug treatment. The latter concept is often called personalized medicine. In this article the application of stable isotope technology in the aforementioned three areas is reviewed with a focus on the developments over the past 25 years. Applications are illustrated with examples from clinical studies in humans.

5.2 Sources of information

We selected articles published in international peer reviewed journals from the Scopus database using the query "stable isotope" combined with "drug", and from our experience in the fields of metabolic research and drug delivery system assessment by stable isotope technology. The main inclusion criterion was that the article covers application of stable isotope technology in humans in relation to drug substances, drug products or drug treatments.

5.3 Principles of stable isotope technology and analysis

Physico-chemical aspects

Stable isotopes are naturally occurring isotopes which differ from their parent atom (the most abundant form of the element) by the presence of one or more additional neutrons. Stable isotopes are not radioactive and may be incorporated in molecules that can be traced by analytical techniques discriminating on molecular weights (table 1). An example of a stable isotopically labeled molecule is ^{13}C-urea as compared to the naturally occurring ^{12}C-urea, having molecular weights of 61.06 and 60.06 g/mol respectively. In this case the mass of the labeled molecule is only modestly increased (1.7%). An extreme situation is $^{2}H_2O$ (heavy water, D_2O) in which 100% of the ^{1}H-atoms is replaced by ^{2}H. The molecular weights of regular water and heavy water are therefore 18 and 20 g/mol respectively; an increase of 11%. Physicochemical properties of heavy water are very different from regular water. For example, the freezing points (3.8°C versus 0.0°C) and the boiling points (101.4°C versus 100.0°C) [3]. Furthermore, when substituting hydrogen for deuterium at a chemically reactive site, enzyme-mediated reaction rates are decreased [4]. Apparently, carbon-hydrogen bonds have lower activation energy than carbon-deuterium bonds. Since biological properties relate to physicochemical properties, the different pharmacological and toxicological aspects of the labeled substance always needs to be considered before starting studies in men.

Safety

Safety concerns of stable isotope labeled drug substances are only of toxicological nature since the isotopes are not radioactive. Toxicity aspects were investigated many years after their discovery and first application in animals and humans because of the limited availability of stable isotope labeled substances [5]. The toxicological properties of stable isotope substitution can be divided into two categories: those that involve deuterium (^{2}H) and those that involve isotopes of higher elements.

Since the mass of ^{2}H is twice that of ^{1}H, labeling may lead to large differences in metabolic handling, depending on the reactivity of the position of ^{2}H in the molecule. Lewis investigated the effect of $^{2}H_2O$ on the development of seedlings from tobacco

Table 1: Naturally occurring stable isotopes used in clinical studies in men

Element	Atomic number	Parent atom	Stable isotope	Abundance (in nature)
Hydrogen	1	^{1}H	^{2}H	0.015%
Carbon	6	^{12}C	^{13}C	1.1%
Nitrogen	7	^{14}N	^{15}N	0.4%
Oxygen	8	^{16}O	^{17}O	0.04%
			^{18}O	0.20%

seed. Seeds in pure heavy water did not germinate in contrast to seeds in regular water [6]. Also, Lewis investigated the effect of heavy water when administered to a mouse in a dose equivalent to 4 to 5 l heavy water to a man. The mouse showed signs of intoxication but did not die. He concluded that "*heavy water is never toxic to any high degree and that it is tolerated in high concentrations by lower organisms. In such cases the rate of the vital processes seems to be roughly proportional to the fraction of the total hydrogen which is* ^{1}H*"*. The mode of toxicity that may be related to an isotope effect is the reduction of the rate of biochemical reactions due to the enhanced mass of a labeled atom or molecule. Because of the great difference in mass between deuterium and hydrogen, a significant isotope effect occurs with deuterium. However, toxicity related to the isotope effect of deuterium can only be produced by very high levels of deuteration (>15% of body water), which highly exceeds the amount of deuterium given in a typical tracer dose of drug [5]. Furthermore deuterium toxicity is only expected when administered as $^{2}H_2O$ since hydrogen of body water is an important source for hydrogen incorporation in organic molecules synthesized by a biological system.

No toxicological studies on stable isotopes of higher elements could be retrieved in the peer-reviewed literature additional to those reviewed by Pons et al. in 1999 [5]. Still, up to the present there is no report on whole organism responses to ^{15}N-enrichment levels. Mayevsky et al. [7] and Samuel and Steckel [8] administered ^{18}O by inhalation and orally (incorporated in drinking water) to mice and found no biological effect. Administration of ^{13}C-labeled endogenous substances, such as carbohydrates and lipids, has been widely applied in biochemical studies but to our knowledge never induced an intoxication, neither when administered as a single oral dose or as continuous intravenous infusion. In practice, when designing an experiment, the dose of a labeled endogenous substance will be chosen between the limit of detection and 10% of the pool size. This upper level is far below toxicological levels. From the information above it can be concluded that toxicological studies with stable isotopes are incomplete and limited. The body of evidence consists of weakly designed preclinical studies supplemented with a large amount of clinical experience with administrations within the context of metabolic studies in animals and humans. The basis for the paradigm of stable isotopes having a margin of safety described as virtually limitless [9] is in our opinion not extensive. However, application of stable isotope technology in humans at historically applied enrichment levels does not pose any clinical relevant risk.

Analysis

In almost all pharmaceutical applications of stable isotopes, isotope analysis has to be performed on drugs, drug-derived metabolites or endogenous substances in complex biological matrices such as plasma or urine. This requires mass spectrometry (MS) if desired in combination with chromatographic separation techniques, such as gas chromatography (GC) or high-pressure liquid chromatography (HPLC) and requires sample preparation (extraction, column separation, hydrolysis) and often also derivatization.

Conventional scanning MS is able to perform measurements at low micromolar concentration levels with sufficient precision when the molar isotope enrichment levels are above 0.1%. When molar isotope enrichments are below 0.1%, isotope ratio mass spectrometry (IRMS) must be applied [10]. This type of MS determines isotope ratios at the atomic level ($^{13}C/^{12}C$, $^{2}H/^{1}H$, $^{18}O/^{16}O$ or $^{15}N/^{14}N$). IRMS originates from geochemistry where minor differences of isotopes at the natural abundance level are to be detected to determine the origin of for instance fossils. An advantage of IRMS is the high precision for the isotope ratio measurement. The major disadvantage is the requirement of higher metabolite concentrations or larger sample volumes compared to scanning MS. For tracer studies as applied in the clinical situation, two types of IRMS may be distinguished: compound-specific and non-compound-specific systems.

Firstly, compound-specific IRMS is characterized by the introduction of compound-specific atoms into the IRMS. The particular compound is first separated from often complex mixtures by GC or HPLC. GC may be coupled directly to the IRMS for breath analysis (e.g. $^{13}C/^{12}C$ in breath CO_2). For this purpose different options are available. Breath may be introduced directly into the IRMS after GC separation of the breath gases (O_2, N_2, CO_2). Recently a non-dispersive infrared technology was developed and applied routinely to measure ^{13}C abundance in breath CO_2.

When nongaseous molecules are to be measured, a GC can be coupled to the IRMS by a capillary reaction interface. The type of interface depends on the atom of interest; for $^{13}C/^{12}C$ a combustion interface, for $^{15}N/^{14}N$ analysis a combustion/reduction interface and for $^{2}H/^{1}H$ and $^{18}O/^{16}O$ analysis a pyrolysis interface is needed. HPLC may be coupled to IRMS by a chemical oxidation interface. So far HPLC-IRMS can only be applied for $^{13}C/^{12}C$ measurements.

Secondly, non-compound specific systems are available, which are characterized by limited sample preparation requirements. One special technique is the combination of elemental analyzer and IRMS interfaced by a combustion/reduction or pyrolysis unit (EA-IRMS). This way $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{2}H/^{1}H$ isotope ratios can be measured in crude biological samples such as urine.

New developments are in progress to use laser infrared technology for these type of measurements. In special cases isotope tracer studies require the measurement of ^{2}H or ^{18}O in body water. In addition to IRMS, laser infrared technology has proved to be very sensitive and accurate for this goal [11].

Availability and quality of labeled substances

Drugs or endogenous substances labeled with various isotopes at specific positions in the molecule can often be purchased from commercial sources. In general, these labeled substances are manufactured by a limited number of companies (Isotec, Cambridge Isotope Laboratories, C/D/N-isotopes). The labeled substances are mostly produced by chemical synthesis and sometimes by biological production methods. Biologically produced labeled substances contain increased levels of a certain isotope due to differences in natural pathways or due to synthesis via a natural pathway which is affected by the incorporation of isotope-labeled precursors. An example of

the first type is corn. Corn is a C_4-plant characterized by a photosynthetic pathway in which more metabolic products enriched in ^{13}C (%^{13}C ~ 1.097%) are generated compared to those from C_3-plants (%^{13}C ~ 1.082%). An example of the second type is to grow plants in a greenhouse with ^{13}C enriched CO_2. As a consequence typical plant products will also be enriched, as ^{13}C enters plant metabolic pathways following $^{13}CO_2$ fixation in the photosynthesis process. Potato starch with 99% ^{13}C-enrichment is produced this way (www.isolife.nl).

Labeled substances are produced in different quality grades. Most companies offer two grades. Firstly, there is the GMP-grade of labeled substances. This grade is produced according to the Good Manufacturing Practice regulations for active pharmaceutical ingredients [12]. These grade substances are sold to be applied in humans. The pharmacist is allowed to use these substances in drug products, based on a risk assessment in which the characteristics of the substance and its application are considered. Quality control consists of verification of documents (status of production site, status of manufacturing process, certificate of analysis) and verification of identity by chemical analysis [13]. Unfortunately, a very limited number of substances is commonly available in the GMP-grade (i.e. ^{13}C-urea and ^{13}C-octanoic acid). Other substances may be produced according to GMP-grade on request but are then very expensive. Secondly, there are the laboratory grade labeled substances. They are produced under general laboratory standards with limited quality control. No stability testing is performed to prove the assigned shelf life. Cleaning procedures of production equipment are not validated. Sometimes, additional microbiological tests are offered on request (e.g. testing for sterility or bacterial endotoxins). These substances are sold with the restriction "not for human use". The pharmacist is allowed to use these substances in drug products, based on a risk assessment in which the characteristics and lower quality assurance of the substance and its application are considered. Quality control consists of verification of documents (status of production site, status of manufacturing process, certificate of analysis), assessment of manufacture-related contaminants and related substances (based on route of synthesis), verification of chemical, physical and microbiological properties per packaged unit and documented support for shelf-life and eventually stability testing [13].

5.4 Assessment of drug pharmacology

Pharmacokinetics

Pharmacokinetics comprises the absorption, distribution, metabolism and elimination characteristics (ADME) of drugs. Pharmacokinetic profiling is conventionally executed with an unlabeled drug by administration, sampling tissues or body fluids of interest and subsequent determination of the drug concentration. From the data obtained, drug-specific pharmacokinetic characteristics can be calculated such as bioavailability, volume of distribution, elimination half-life, clearance and protein binding. Kinetic

studies with stable isotope labeled drug substances are generally not performed. It is only the preferred option when conventional pharmacokinetic profiling of a drug is not feasible. This is the case in the following three situations, which are discussed in more detail below:

» studies on pharmacokinetics under chronic administration of the same drug;
» studies of the pharmacokinetics of high-clearance drugs;
» studies on the metabolism of drugs yielding metabolites that also occur naturally as endogenous substances.

Firstly, when medical treatment demands chronic administration of a drug, a change in drug pharmacokinetics may occur because of time- (carbamazepine, acetaminophen) or dose-dependent (phenytoin) kinetics. When discontinuation of treatment is not acceptable, administration of a single dose of stable isotope labeled drug may be the way of choice to determine the actual pharmacokinetic parameters in a patient. A typical example is the study by Malik et al [14], in which the pharmacokinetic profiles of phenobarbital and phenytoin were determined in five respectively three neonates who were treated with those drugs at that time because of their history of seizures. A single dose of 2-^{15}N-^{13}C-phenobarbital or 2-^{13}C-1,3-^{15}N-phenytoin was administered orally together with a reduced intravenous dose of unlabeled drug product. Pharmacokinetic parameters as clearance, half-life and volume of distribution could be calculated from drug plasma concentrations as a function of time. A large variation in pharmacokinetic parameters was observed (coefficient of variation ~ 50%), which underlines the necessity of therapeutic drug monitoring for phenobarbital and phenytoin.

Secondly, stable isotopes may be used to correct for the influence of day-to-day variation in clinical studies with multiple day administration and sampling. This strategy is especially of added value for high-clearance drugs. A nice example is the study of Bode et al. [15] who investigated nifedipine absorption in different regions of the gastrointestinal tract. On separate occasions, nifedipine solution was administered locally into the stomach, the small intestine and two sites in the colon in four healthy male volunteers using a remote controlled drug delivery device (high frequency triggered capsule). In order to assess absolute and relative bioavailabilities, an intravenous infusion of nifedipine was given on a separate occasion and all treatments were accompanied by a simultaneous oral dose of a $^{13}C_4$-nifedipine solution to mathematically correct for day-to-day pharmacokinetic variation. The stable isotope labeled substance was used in this study as an internal standard. A comparable approach was used by Fromm et al [16] to investigate the presystemic gut wall elimination of verapamil.

Thirdly, studying drug metabolism is conventionally performed by measuring the parent substance and its metabolites. This approach works well as long as the metabolites are not naturally present. This problem of drug metabolites being endogenous substances as well, was encountered by Durso et al [17]. They investigated how carbidopa, a dopa-decarboxylase inhibitor, affects peripheral

levodopa pharmacokinetics. They used 1,2,3,4,5,6-^{13}C-levodopa as drug substance, in which all ^{12}C atoms of the phenyl-group were replaced by ^{13}C. Levodopa is metabolized by dopa-decarboxylase to yield dopamine which in turn is converted into homovanillic acid (HVA) by monoamine-oxidase and catechol-O-methyl-transferase. Both dopamine and HVA naturally occur in the human body, where dopamine acts as a neurotransmitter. Dopamine and HVA derived from the labeled levodopa still contained the labeled phenyl group and could thus be discriminated from naturally occurring (unlabeled) counterparts. This approach of metabolic tracing may also be well suited to study the metabolism of biotech-drugs such as peptides and proteins. The only clinical study in men available on this subject, is the work by Ligthart-Melis et al [18] who investigated the influence of the route of administration (enteral or intravenous) of L-(2-^{15}N)-glutamine on its metabolic fate. An important topic, since glutamine and its metabolite arginine are indicated to be an essential part of the immunonutrition of seriously ill patients [19]. It was shown that enteral compared to intravenous L-(2-^{15}N)-glutamine results in lower arterial plasma ^{15}N-glutamine, higher plasma ^{15}N-citrulline and equal ^{15}N-arginine concentrations. In a follow up study [20] the authors investigated the same metabolic pathway, but now for oral or intravenous alanyl-(2-^{15}N)-glutamine (Dipeptiven®). Again, enteral administration was shown to contribute more to *de novo* synthesis of arginine, which stimulates the immune response of patients with critical illnesses (immunonutrition).

In the two earlier reviews from more than two decades ago, racemate kinetics, tissue distribution and studying deep pool effect have been suggested as additional applications for stable isotope technology, but these have not gained extensive follow-up. For these applications reference is made to earlier reviews [2,21].

In conclusion, pharmacokinetic profiling is generally performed by studying the unlabeled drug substances in the body. This approach has less complexity, general availability of facilities and lower cost, compared to applying stable isotope labeled drug substances, Stable isotope labeled drug substance profiling is only the preferred option when the conventional approach is not feasible.

Mechanism of action

Pharmacodynamics often involves binding of drug substances to targets such as receptors, enzymes, ion channels, transporters or nucleic acids followed by signal transduction leading to a response. A great challenge is to understand the role of these drug targets in relation to physiology and pathology and their interaction with other drug targets and cofactors. Drugs are meant to interfere in pathological pathways by blocking or activating specific targets. In clinical pharmacology these pathways are confirmed and the real functionality of a drug in these pathways needs to be tested. Pharmacodynamics research approaches show a wide variety, mainly depending on the class of drugs being investigated. Apart from conventional biochemical testing, stable isotope labeled substances are widely applied to dynamically investigate metabolic processes. Stable isotope technology can be an excellent tool to investigate the mechanism of action of drug substances.

A nice example is given by the efforts to reduce cholesterol levels in hypercholesterolemia patients. Drug targets are enzymes in cholesterol metabolism, such as HMG-CoA reductase in cholesterol synthesis (inhibited by statins) or the sterol transporter Niemann-PickC1-Like1 in cholesterol absorption (inhibited by ezetemibe) or cholesterol catabolism (increase via enhanced bile salt formation by bile salt sequestrants_as cholestyramine, colestipol, colesevelam). Functional tests to measure cholesterol synthesis, cholesterol absorption and bile salt synthesis are based on isotope incorporation (cholesterol synthesis) and isotope dilution (cholesterol absorption and bile salt synthesis) [22]. Originally tests have been developed with radioactive isotopes (^{14}C, ^{3}H). Later on, they were modified into methods applying stable isotopes (^{13}C and ^{2}H). Cholesterol synthesis is measured by a constant intravenous infusion of ^{13}C-acetate which is in the form of acetyl-CoA the building block of cholesterol. Cholesterol absorption is measured via oral administration of ^{13}C or ^{2}H-cholesterol.

Two techniques have been developed. One relates the fecal excretion of labeled cholesterol to that of labeled sitostanol. Sitostanol is a non-absorbable sterol and the malabsorbed fraction is considered 100%. The ratio of labeled cholesterol to labeled sitostanol in feces reflects the malabsorbed fraction of the orally administered labeled cholesterol. The second technique applies an additional differently labeled cholesterol that is administered intravenously at the same time as the first one is administered orally. The ratio of the two labeled versions of cholesterol is determined in plasma after certain time intervals. This ratio represents the absolute bioavailability of orally administered (labeled) cholesterol. To measure bile acid synthesis, ^{13}C- or ^{2}H- bile acids were administered orally and plasma samples were collected over four days. The decay of label represents the fractional turnover rate (FTR, day^{-1}), whereas from the intercept at t=0 the pool size can be calculated (micromole). Multiplying pool size and FTR results in the daily turnover rate, which equals the synthesis rate. It is of interest to realize that treatment with bile acid sequestrates does not only increase bile acid synthesis. As a consequence of treatment the hepatic cholesterol pool becomes reduced leading to an increased cholesterol synthesis. Therefore, most often bile acid sequestrant therapy is combined with statin treatment in order to maximize the clearance of cholesterol from the plasma.

Atorvastatin is known to reduce total cholesterol, triglycerides and LDL cholesterol. It were Chan et al [23] who hypothesized that atorvastatin may decrease chylomicron remnant concentrations by increasing their metabolism. Rate of metabolism of a remnant-like intravenous emulsion labeled with cholesteryl-^{13}C-oleate was determined by measuring breath $^{13}CO_2$ and compared to placebo. Atorvastatin was shown to increase the rate of metabolism of this remnant-like emulsion.

Fibrates have been introduced for the treatment of hypertriglyceridemia. The lipid reducing mechanism of action is the interaction in the lipoprotein metabolism by affecting the apoB synthesis and VLDL secretion. To show the mechanism of action *in vivo* in humans apoB metabolism has been studied using stable isotopes. ^{13}C-valine or other labeled amino acid was infused and the incorporation of ^{13}C-valine in VLDL particles isolated from plasma samples was measured [22,24].

The use of pre- and probiotics is promoted commercially in order to improve intestinal functions. One positive effect is nitrogen uptake reduction from the colon [25]. The mechanism of this effect has been studied by the oral administration of lactose-^{15}N-ureide. This substance is not absorbed from the small intestine but fermented in the colon leading to production and subsequent absorption of ^{15}N-ammonia. The results demonstrate the suppression of the generation and accumulation of ^{15}N-ammonia and other toxic fermentation products during treatment with pre- and probiotics [25].

The drug product acarbose interacts with the digestion of starch by inhibiting the intraluminal enzyme α-glycosidase. It is recommended in the treatment of diabetes type 2 and acts by partial inhibition of starch digestion leading to a slower influx of glucose from the intestine into the circulation. The mechanism of action has been investigated by applying naturally ^{13}C-enriched corn pasta with and without acarbose treatment [26] and ^{13}C-sucrose [27]. Starch digestion, sucrose digestion and availability of glucose were shown to be reduced.

Premature infants have diminished synthesis of the surfactant phosphatidylcholine (PC). In vitro studies showed that (prenatal) corticosteroids and exogenous surfactants stimulate the synthesis of PC. Bunt et al. showed that intramuscular betamethasone 12 mg [28] and exogenous surfactants [29] stimulate PC synthesis in premature infants with respiratory distress syndrome. Infants received a 24 h-infusion with stable isotope $^{13}C_6$-glucose starting around 5 h after birth. The ^{13}C-incorporation into palmitic acid in surfactant PC was measured. Per dose of prenatally administered corticosteroids the synthesis rate increased by 40% [28]. Surfactant production has also been studied using intravenous infusion of labeled fatty acids [30].

In conclusion, biochemical testing, in combination with stable isotope technology, is a valuable tool to study pharmacodynamics of a drug product.

Mechanism of toxicity and side effects

Metabolism-mediated side effects are often discovered clinically and explained by changed plasma levels of endogenous substances or drug-derived metabolites. However, knowledge on the mechanism of toxicity and side effects on a molecular level is limited [31]. For example, while troglitazone was withdrawn from the market in 2000 because of serious hepatotoxicity and replaced by chemical analogues, the mechanism of toxicity has up till now not been elucidated yet [32].

The link between drug-derived metabolites and organ toxicity may be studied by modulating drug metabolism. Enzyme-mediated drug metabolism can be modulated by deuteration of drug substances. Advent of organ toxicity may be compared between labeled and unlabeled drug substances to investigate a causal relation. In fact, use is made in these studies of the isotope effect of changed reaction rates (see 3.2). Further detailed insight may be generated by selective deceleration of certain metabolic pathways by selective replacement of hydrogen for deuterium. The relative amounts of different metabolites generated may be changed and related to different toxicity profiles [31].

When the drug target is known to be involved in other metabolic pathways, toxicity and type A-side effects may be predicted to a certain extent. The mechanisms causative of changes in plasma levels of endogenous substances can only be unraveled with biochemical tests; alone or in combination with stable isotope technology. As an example, we discuss the side effects of parenteral nutrition. It is generally known that long term parenteral nutrition may lead to intrahepatic cholestasis [33]. Using stable isotopes (^{13}C-methionine) it was shown that hepatic mitochondrial function became reduced [34]. Other metabolic effects of parenteral nutrition related to hepatic function included increased cholesterol synthesis and reduced bile acid synthesis. The latter was proven by stable isotope facilitated measurement of the plasma concentration of 7α-hydroxy-4-cholesten-3-one, an intermediate in bile acid synthesis indicative for rate of synthesis [35]. The changes in cholesterol and bile acid metabolism were explained by the fact that the gall bladder remains unstimulated when food is not administered enterally. This leads to reduced cholesterol absorption and a feedback regulation of cholesterol and bile acid synthesis. In studies using $^{2}H_{5}$-phenylalanine it was demonstrated that albumin production in the liver is increased during parenteral nutrition, whereas at a whole body level protein catabolism is reduced [36]. This implies a direct effect on hepatic protein metabolism. Interestingly, similar effects on protein metabolism have been documented during the treatment with infliximab [36].

It has been shown with stable isotope labeled amino acids that cyclosporine A treatment after liver transplantation leads to hyperlipidemia due to interference with lipoprotein metabolism. Hepatic bile acid synthesis is reduced due to cyclosporine A treatment as has been shown with stable isotope bile acid kinetic studies in rats [37] and humans [38].

In conclusion, stable isotope technology is highly suitable to investigate the mechanism of toxicity and side effects of drug substances. Labeled drug substances may be used to study drug metabolism-mediated toxicity. Labeled endogenous substances may be used to study the mechanism underlying changed plasma levels of endogenous substances. An increased application of stable isotope technology in these types of studies is envisaged, since drug safety is still gaining interest and toxicity a major cause of market withdrawal of drugs [39].

5.5 Assessment of drug products and drug delivery systems

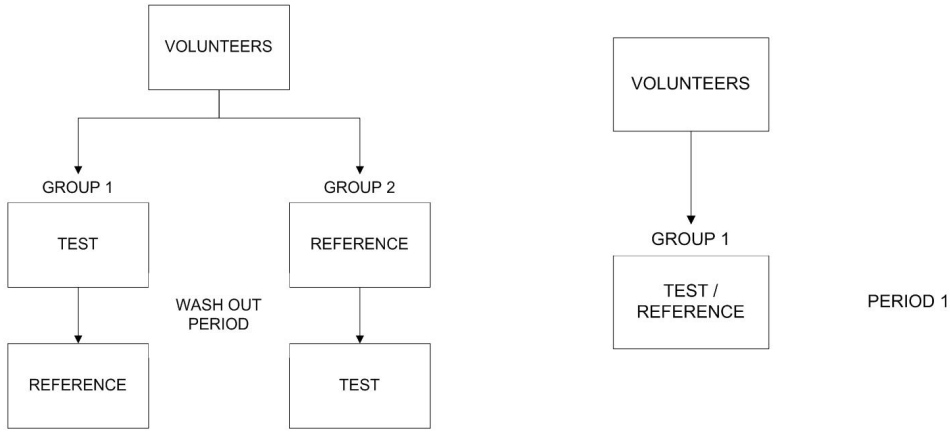
Bioavailability

A key characteristic of a drug product intended to treat a systemic condition, is the ability to deliver the drug substance to the systemic circulation in an amount sufficient to elicit the desired response. This characteristic is called bioavailability and captures two features, namely rate and extent of absorption. Most bioavailability studies are of a comparative nature. This means that they compare the bioavailability of the test product to that of a reference. The reference product may be administered

intravenously or non-intravenously. The fractions reaching the systemic circulation relative to the reference are called the absolute or relative bioavailability, respectively. Conventionally a clinical study has a randomized two-period, two-sequence crossover design (figure 1a). An adequate wash-out period between subsequent periods is needed to avoid drug carry-over effects. The validity of this study design is based on the assumption that distribution, metabolism and clearance of the particular drug remain constant over time. However, for drugs that are subject to extensive and highly variable metabolism this assumption may be invalid. An alternative to the conventional approach is the application of the stable isotope technology, in which the test and the reference doses are administered concomitantly, thereby minimizing the influence of intra-individual variability of kinetics. Consequently, the clinical study design can be shortened to a randomized one-period, one-sequence crossover design (figure 1b). Our literature search revealed 53 peer-reviewed papers describing investigations into the bioavailability or release profile of drug products applying stable isotope technology (table 2). As can be seen, the determination of the absolute bioavailability is an important application of stable isotope technology in this category of studies (25 out of 52 papers). Strong et al [40] were the first to describe this approach, in 1975. They determined the absolute bioavailability of oral N-acetylprocainamide (NAPA) by comparing its kinetics to that of co-administered ^{13}C-NAPA intravenous injection. Since then the absolute bioavailability has been determined for formulations containing a wide variety of drug substances. Special applications are the determination of the absolute bioavailability of separate enantiomers forming a racemate (R- and S-forms of verapamil and ibuprofen) and the studies on intestinal absorption windows (nifedine and verapamil).

The stable isotope technology has also been used to determine the relative bioavailability of a dosage form (13 from 49 papers) A typical example is the study by Shibuya et al [49] in which the bioavailability of a stabilized formulation of a nitroglycerin sublingual tablet (excipient = methylcellulose) was compared to the conventional formulation (excipient = lactose). A conventional tablet containing $^{15}N_3$-nitroglycerin was administered at the same time as the stabilized tablet containing regular nitroglycerin. In a one-day experiment it could be shown that both formulations exhibited equal bioavailability.

An interesting study design has been applied by Gammans et al [46]. The bioavailability of trazodone from a new tablet formulation (dividose) was compared to the marketed tablet formulation (film-sealed tablet). An open three-period crossover design was applied in which the dividose, a film-sealed tablet or a solution of trazodone was given. In addition, each subject received 5 ml of a 1% solution of $^{2}H_4$-trazodone concomitantly with each formulation. The addition of the stable isotope labeled trazodone revealed additional information. Firstly, it became possible to determine intra-individual variability, which turned out to be 6-38%, being less than inter-individual variability (26-55%). Secondly, the ability to demonstrate dosage form equivalence could be enhanced by normalizing the data using the results obtained with the concomitantly administered $^{2}H_4$-trazodone. This was demonstrated



1a: A two-period, two sequence, crossover study design

1b: A one-period, one sequence, crossover study design

Figure 1: Schematic representation of the study design of a comparative bioavailability study with (a) or without (b) stable isotope technology

by the relatively smaller 95% confidence intervals and increased power for the relative AUC data compared to the trazodone AUC data alone. Thirdly, the equal plasma concentrations at all time points of trazodone and $^{2}H_{4}$-trazodone administered as a solution, indicated that the introduction of a stable isotope label does not alter trazodone pharmacokinetics. With this result absence of the isotope-effect had been reconfirmed which is an important assumption supporting this type of clinical pharmaceutical investigation.

In conclusion, stable isotope technology may be used to determine absolute and relative bioavailability drugs, their racemates and enantiomers. While acknowledging publication bias, the place for this technology in bioavailability studies seems limited as is concluded from the 49 peer-reviewed papers that could be retrieved. When bioavailability has to be measured under steady-state conditions or when drug substance pharmacokinetics is known to exhibit large inter- and intrasubject variations, stable isotope technology seems to be the appropriate method. In other situations, the bioavailability study should be performed straightforward with unlabeled drug substances.

Release profile of drug delivery systems

To achieve an optimal therapeutic effect, some drug substances can be administered by a drug delivery system with modified-release properties. Objectives that are aimed for by the modified release profile encompass a modification of the rate and extent at which the drug substance becomes available at the site of action, the maintenance of therapeutic blood levels for prolonged period of time or the prevention of toxic blood concentrations. For many oral modified-release products, drug delivery occurs in intestinal segments distal from the stomach (e.g. the duodenum, jejunum, ileum or

colon). In vivo determination of the precise segment of release is the ultimate proof of a valid drug delivery system. The golden standard for these investigations is imaging. In the international literature a myriad of approaches are described to image the release profile of modified-release systems, for example endoscopy, radiology, gamma scintigraphy and magnetic resonance imaging. These technologies are in most cases combined with pharmacokinetic data. Currently, gamma scintigraphy is considered to be the golden standard. To perform the clinical study a special formulation is manufactured in which enriched samarium oxide ($^{152}Sm_2O_3$) is incorporated. Then, the drug product is exposed to a neutron flux, that transforms stable ^{152}Sm into radioactive ^{153}Sm with a half-life of 46 hours. ^{153}Sm emits gamma radiation which can be detected in the body by a gamma camera [89]. Localization and monitoring of the radiolabel led drug product inside the body can be performed.

A more recently expedited application of stable isotope technology is to determine the release profile of these modified-release drug delivery systems in vivo. When the release profile of a formulated drug product with modified-release technology incorporated has to be assessed, regular bioavailability studies may be performed with or without stable isotope technology. Wilding et al. [61] determined the release profile of carbamazepine formulated in an osmotically controlled release oral delivery system (OROS). The study design consisted of conventional pharmacokinetics with gamma scintigraphy to localize the drug delivery system. Carbamazepine OROS was administered concomitantly with ^{15}N-carbamazepine oral suspension. A one day study design was chosen because of the long elimination half life (up to 36 h) of carbamazepine which would necessitate a wash-out period of 7 days. Carbamazepine OROS showed a reduced bioavailability (69%) compared to carbamazepine as a suspension. The fraction of the dose recovered was 16.8%. Systemic absorption from the colon proved to be reduced compared to absorption from the upper gastrointestinal segments (stomach, small intestine).

In case a drug delivery system as such needs to be assessed in vivo, a stable isotopically labeled substance may be used which provides information regarding the intestinal segment of release. Stable isotope technology has been used to assess oral colon drug delivery systems in a few cases. For this goal ^{13}C-glucose [71,86] and ^{13}C-urea [82,88] have so far been used as marker substances. Both marker substances signal their arrival in bacteria-rich intestinal segments. Bacterial ureases transform ^{13}C-urea into ammonia and $^{13}CO_2$ by urease and ^{13}C-glucose is fermented to yield $^{13}CO_2$, ^{13}C-short-chain fatty acids and H_2O. In both cases $^{13}CO_2$ is partly (about 55%) absorbed into the systemic circulation, transferred to the lungs and exhaled via breath [87]. The appearance in breath signals almost real time the release in the colon because of the very short transfer time of CO_2 from the colon lumen to the breath (< 5 minutes [87]). The marker substance of first choice for assessing oral colon delivery systems is ^{13}C-urea. It has good physicochemical characteristics and an excellent safety profile. Urea is freely soluble in water (1 g/mL) and permeates rapidly through the intestinal wall into the systemic circulation with virtually no presystemic degradation. Secondly, the distribution of ^{13}C-urea is favorable. The

Table 2: Papers describing the application of stable isotope technology to assess the bioavailability or release profile of drug products and delivery systems

Author	Year	System (reference)	Labeled substance	System (test)	Study objective[1]	Reference
Strong et al	1975	Intravenous injection	^{13}C-N-acetylprocainamide	Capsule (immediate release)	ABA	40
Heck et al	1979	Oral solution	$^{2}H_2$-Imipramine	Tablet (immediate release)	RBA	41
Eichelbaum et al.	1981	Intravenous infusion	$^{2}H_3$-Verapamil	Oral solution	ABA	42
Eichelbaum et al.	1981	Oral solution	$^{2}H_3$-verapamil	Tablet (prolonged release)	RBA	43
Meresaar et al.	1981	Intravenous injection	$^{2}H_2$-methadone	Tablets	ABA	44
Rigby et al	1983	Pro-Banthine 15 mg	Propantheline bromide	Tablet (new formulations)	RBA	45
Gammans et al	1984	Oral solution	$^{2}H_4$-Trazodone	Tablet (immediate release)	RBA	46
Vogelgesang et al	1984	Intravenous injection	$^{2}H_7$-Verapamil		ABA	47
Marvola et al	1985	Tablet (extended release)	$^{2}H_7$-Verapamil	Tablet (extended release)	RBA	48
Shibuya et al	1985	Tablet (excipient lactose)	$^{15}N_3$-Nitroglycerin	Tablet (excipient methylcellulose)	RBA	49
Hatch et al.	1986	Oral solution	$^{2}H_3$-verapamil	Tablet (immediate release)	RBA	50
Fujioka	1987	Endogenous testosterone	$^{2}H_3$-17α-Testosterone	Intramuscular injection	RP	51
Looareesuwan et al.	1987	Oral solution	^{2}H-mefloquine	Tablet (immediate release)	RBA	52
Mikus et al.	1987	Intravenous injection	$^{13}C_4$-Nitrendipine	Oral solution & Tablet (immediate release)	ABA	53
Hallen et al.	1988	Tablet	$^{2}H_2$-terodiline	Tablet	RP	54
Atkinson et al.	1989	Intravenous injection	^{13}C-N-acetylprocainamide	Capsule (immediate release)	ABA	55

Fujioka	1989	Endogenous testosterone	$^{2}H_{3}$-17α-Testosterone proprionate	Intramuscular injection	RP	56
Shinohara et al	1990	Solution	$^{2}H_{3}$-17α-Testosterone	Tablet	RBA	57
Aronof et al.	1991	Intraveous infusion	$^{15}N_{2}$-cibenzoline	Capsule (immediate release)	ABA	58
Benowitz et al	1991	Intravenous infusion	$^{2}H_{2}$-Nicotine	Capsule (immediate release)	ABA	59
Benowitz et al	1991	n.a.	$^{2}H_{2}$-Nicotine	Transdermal delivery system	RP	60
Wilding et al	1991	Intravenous infusion Oral suspension	^{15}N-Carbamazepine	Tablet (extended release) Tablet (extended release)	ABA RP	61
Bredberg et al	1992	Intravenous infusion Oral solution	$^{2}H_{6}$-Terbutaline	Tablet Tablet	ABA RBA	62
Browne et al	1993	Intravenous infusion (sodium salt of phenytoin)	$^{15}N^{2}$-$^{13}C_{3}$-Phenytoin (sodium salt) and 13C3-phenytoin phosphate	Intravenous infusion (phosphate-ester of phenytoin)	ABA	63
Hall et al.	1993	Intravenous injection	S-$^{2}H_{4}$-ibuprofen	Tablet (immediate release) & Intravenous injection	ABA	64
Houezec et al.	1993	n.a.	$^{2}H_{2}$-nicotine	Subcutaneous injection	RP	65
Richard et al	1994	Tablet (immediate and slow release)	H_{2}-Metoprolol	Tablet (extended release)	ABA	66
Hage et al	1995	Oral solution	$^{2}H_{3}$-Flecainide	Tablet (controlled release)	ABA	67
Sun et al	1995	Intravenous infusion	^{15}N-Nitroglycerin	Transdermal delivery system	ABA	68
Voortman et al	1995	Intravenous infusion	^{13}C-Mirtazapine	Tablet (immediate release)	ABA	69
Bode et al	1996	Intravenous infusion	$^{13}C_{4}$-Nifedipine	Intrajenunal and –colonic delivered solution	ABA	70
Cummings et al	1996	n.a.	^{13}C-glucose	Capsule (delayed release)	RP	71
Gross et al.	1997	Capsule (immediate release)	S-$^{2}H_{2}$-gallopamil	Capsule (immediate release)	RBA	72
Benech et al.	1998	Intravenous injection	^{25}Mg and ^{26}Mg	Tablets	ABA	73

Table 2: Continued

Author	Year	System (reference)	Labeled substance	System (test)	Study objective[1]	Reference
Fromm et al.	1998	Intravenous injection	$^{2}H_{7}$-Verapamil	Tablet (prolonged release)	ABA	74
Dilger et al	1999	Intravenous infusion	$^{2}H_{7}$-Verapamil	Tablet (prolonged release)	ABA	75
Pieniaszek et al	1999	Oral solution	$^{13}C_{6}$-Moricizine	Tablet (immediate release)	RBA	76
Yeh et al.	1999	Intravenous injection	$^{2}H_{6}$-Indinavir	Capsule (immediate release)	ABA	77
Gross et al.	2000	Capsule (immediate release)	S-$^{2}H_{2}$-gallopamil	Capsule (immediate release)	RBA	78
Heikkinen et al.	2001	Intravenous injection	^{13}C-Entacapone	Oral solution	ABA	79
Glaeser et al.	2004	Intravenous injection	$^{2}H_{7}$-Verapamil	Intrajenunal delivered solution	ABA	80
Kasuya et al.	2005	Intravenous injection	$^{2}H_{10}$-Phenytoin	Tablet	ABA	81
Verbeke et al	2005	n.a.	^{13}C-urea / ^{15}N-urea	Capsule (delayed release)	RP	82
Foster et al.	2006	Intravenous injection	$^{2}H_{6}$-methadone	Oral solution	ABA	83
Majumdar et al	2006	Intravenous infusion	$^{13}C_{2}$-$^{15}N_{3}$-Aprepitant	Capsule (immediate release)	ABA	84
Schulz et al.	2006	Intravenous injection	^{13}C-dichloroacetate	Oral solution	ABA	85
Schellekens et al	2008	Capsule (immediate release)	^{13}C-glucose	Capsule (delayed release)	RP	86
Schellekens et al.	2009	Capsule (immediate release)	^{13}C-urea	Capsule (delayed release)	RP	87
Schellekens et al	2010	Capsule (immediate release)	^{13}C-urea	Capsule (delayed release)	RP	88

[1] ABA = absolute bioavailability, RBA = relative bioavailability, RP = release profile, n.a. = not applicable

volume of distribution was ~0.6 L/kg indicating uniform distribution over the water compartment in the human body [92]. Thirdly, ^{13}C-urea may be absorbed from the gastro-intestinal tract in intact form or fermented, depending on the presence and activity of ureases (generated by bacteria in the colon flora). Since urea is the end product of nitrogen physiology, no relevant amount of metabolite is formed after intact absorption. Fourthly, urea is eliminated mainly by renal excretion. This enables easy (non-invasive) sampling to quantify the amount of absorbed intact ^{13}C-urea. Fifthly, urea exerts no pharmacologic effects, making it a very attractive marker for clinical trials, especially in children or healthy volunteers.

In conclusion, stable isotope technology may be a valuable tool in the assessment of modified-release oral drug delivery systems. The labeled substance may be the drug substance itself (formulated as intravenous injection or oral solution serving as a reference) or a labeled tracer that signals the site of release. Compared to imaging by gamma scintigraphy, stable isotope technology has two distinct advantages. One, trial subjects are not exposed to radioactive radiation. Two, exposing the drug delivery device to a neutron flux to activate ^{152}Sm, elicits unknown and unpredictable effects on the release profile. This compromises the external validity of the test. On the other hand, there is only one stable isotopically labeled tracer available that has been validated for a specific intestinal segment. For oral colon delivery devices, ^{13}C-urea may be used.

5.6 Assessment of patients

Phenotyping

It is generally accepted that certain drug treatments, such as thiopurines (azathioprine and 6-mercoptopurine) [90] and cytostatics, should be pre-adjusted to the characteristics of the individual patient in order to improve drug efficacy or to reduce toxicity and side effects. The concept of personalized medicine is nowadays mainly practiced by dosing on the basis of weight or body surface or by monitoring drug concentrations in plasma and subsequent dose adjustments [91,92]. Personalized medicine increases momentum because of an increasing number of geno- and phenotyping tests aimed at predicting drug metabolism. These tests would enable physicians to rapidly identify responders or non-responders to various drugs prior to initiation of therapy. The CYP-enzymes are an important subset of phase I drug metabolizing enzymes. With the information on CYP-enzyme activity, physicians and clinical pharmacists are able to ensure administration of the right drug, at the right dose, at the right time, to the right individual to obtain the right clinical outcome. Stable isotope technology can be used to provide rapid *in vivo* phenotype assessment of CYP-enzymes. The CYP1A2 activity may be assessed by ^{13}C-methacetin breath test and is relevant for drug and xenobiotic metabolism. The CYP2D6 activity may be assessed by ^{13}C-dextromethorphan breath tests and is relevant for the metabolism of tamoxifen, β-blockers, antiarrhythmics, antidepressants, antipsychotics and opiates,

CYP2C19 activity may be assessed by ^{13}C-pantoprazole breath test and is relevant for the metabolism of proton pump inhibitors, clopidogrel, cyclophosphamide and thalidomide [93]. Currently, application of these breath tests in clinical practice is limited. An interesting upcoming application is the development of a breath test using 2-^{13}C-uracil to determine dihydropyrimidine dehydrogenase (DPD) enzyme activity. DPD metabolizes 5-fluorouracil (5-FU), an anticancer agent with a narrow therapeutic window. DPD deficiency is an inborn genetic disorder that may lead to severe toxicity or even death [94] in patients treated with 5-FU. Screening for DPD deficiency is therefore recommended before 5-FU-based treatment is started. Currently, the American Food and Drug Administration (FDA) issued a contraindication for the use of 5-FU topical cream for patients with known DPD deficiency. The European Medicines Agency (EMA) demands intensive monitoring when this cream is prescribed to DPD deficient patients. Currently, genotyping is hampered by insufficient knowledge on identity and clinical relevance of mutations and their interactions. The remaining alternative is phenotyping, i.e. determination of DPD activity [94]. Van Staveren et al. [95] showed that a plasma-based uracil screening test can differentiate between patients with deficient or normal DPD-activity based on the uracil/dihydrouracil ratio in plasma. Mattison et al. [96,97] showed that breath $^{13}CO_2$ appearance after administration of 2-^{13}C-uracil parallels plasma 2-^{13}C-uracil and 2-^{13}C-dihydrouracil pharmacokinetics and is an accurate measure of inter-individual variation in DPD activity. Currently, none of the approaches has gained the preference. Information on the sensitivity, specificity, positive predictive value and cost of all methods is incomplete. In our opinion, phenotyping may gain the preference in the near future supported by the current inadequacy of the genotype-phenotype correlation. Additional and unresolved problems are phenotype changes during the course of life (e.g., concurrent medication use, environment, age) that are not taken into account by the genetic profile.

Monitor drug treatment effect

Monitoring of drug treatment effects is an essential part of medical treatment. Efficacy or toxicity of a drug may be indicated by the presence or amount of biochemical metabolites. As described in paragraphs 4.1.2 and 4.1.3, biochemical testing by stable isotope technology enables the dynamic assessment of metabolic pathways. As such, the test may be used in medical diagnosis. The same test can also be used to determine the relationship between drug treatment, changes in pathological processes and ultimately the efficacy of a drug product. In most cases, also approaches not applying stable isotope technology may be suitable. A choice is made based on test-specific characteristics. In general, stable isotope biochemical testing is an approach that scores very well on aspects as patient safety, comfort (i.e. non-invasive sampling) and costs, while performing equally on specificity and sensitivity in comparison to plasma- or biopsy-based testing.

An important example of a test applied in medical diagnosis and monitoring drug treatment effect is the ^{13}C-urea breath test. It allows the detection of Helicobacter

Table 3: Products containing stable isotopically labeled substances to be used in clinical trials or medical practice; regulatory requirements to assure quality of design and clinical production

Product class		Quality assurance for product design (to be included in clinical trial application)	Quality assurance for Clinical Production		
			Manufacturing License	Pharmacy	HACCP[1]
Medicinal product	IMP[2]	Describe design, clinical production and (pre) clinical pharmacology and toxicology of IMP conform the format of an IMPD[3]	+	-	-
	Magistral or officinal preparation, NIMP[4]	Describe design, clinical production and (pre) clinical pharmacology and toxicology of IMP.	-	+	-
Medical device		Describe design and clinical production of the medical device.	-	+	-
Food product		Describe design and clinical production of the food product.	-	-	+

[1] Hazard Analysis and Critical Control Points; [2] Investigational Medicinal Product; [3] Investigational Medicinal Product Dossier; [4] Non-Investigational Medicinal Product

pylori (Hp) infection in the stomach [98]. It shows the presence (or absence) of Hp urease which converts orally administered ^{13}C-urea to $^{13}CO_2$ and ammonia. Since $^{13}CO_2$ is partially expired by breath, ^{13}C in breath signals Hp presence in the stomach. Upon detection of Hp, antibiotic eradication therapy is initiated. The same ^{13}C-urea breath test is subsequently applied to monitor drug treatment efficacy, since absence of ^{13}C in breath indicates eradication of Hp and successful therapy. There are many ways of testing: serology, ^{13}C-urea breath test, and fecal antigen test. Compared to histology it is estimated that the ^{13}C-urea breath test is 95% sensitive and 95% specific when diagnosing Hp infections [99]. Elwyn et al [100] performed a cost effectiveness analysis and concluded that serology is less accurate than the ^{13}C-urea breath test, and fecal antigen test, because it is not able to distinguish between present and past infections. The fecal antigen test should according to Elwyn et al. be preferred over the ^{13}C-urea breath test because the breath test is "cumbersome to perform".

Gastric emptying may be investigated in the clinical setting in case of weight loss, suspicion of mechanical or anatomical obstructions, altered function due to underlying disease (e.g. amyloidosis), nausea or vomiting. Gastric emptying tests are applied for medical diagnosis as well as for monitoring the effect of drug treatment. Gastroparesis is treated by acceleration of gastric emptying with cisapride. The symptoms of dumping syndrome are treated with octreotide (inhibition of release of insulin) or acarbose, (inhibition of α-glycoside hydrolase) to moderate postprandial hypoglycemia and early hyperglycemia, respectively. Diagnosis of impaired gastric emptying and monitoring of drug treatment effects are traditionally performed by gammascintigraphy. Recently however, two stable isotope breath tests have been introduced for this purpose, one with sodium-^{13}C-acetate [101] to measure gastric emptying of liquids and one with ^{13}C-octanoic acid [102] to measure gastric emptying of solids. Braden et al [101] investigated the effect of cisapride drug treatment on gastroparesis and found that the half emptying times in diabetic gastroparesis became improved from 4 to 3 hours. Also dyspepsia improved, while control of glycemia did not.

Pancreatic enzyme replacement therapy is prescribed in case of exocrine pancreatic insufficiency to avoid malnutrition [103]. Dysfunction of the digestive enzymes lipase, protease and α-amylase are difficult to assess. The ideal test to assess pancreatic enzyme function does not exist. Breath tests have been developed [101] and are based on oral intake of ^{13}C-mixed triglyceride (test lipase activity), cornstarch (α-amylase activity) or ^{13}C-egg protein (proteases activity). An alternative method to detect fat malabsorption is the performance of a fat balance. For this purpose the intake of fat and the fecal excretion of fat are measured. This requires fecal collection over a 3-day time period. The fecal collection and fecal fat analysis are unpleasant tasks for the patient and analytical personnel.

Lactase enzyme supplements are prescribed in case of lactose intolerance as a more attractive option than dietary restrictions having nutritional disadvantages [104]. Lactase activity and effect of supplementation is generally assessed via the combined ^{13}C-lactose / hydrogen breath test [105]. This test has limited accuracy

due to false positive outcomes. The plasma ^{13}C-lactose / $^{2}H_2$-glucose test however, showed excellent accuracy (no false negatives), but unfortunately is too complex for routine patient care [105]. In conclusion, stable isotope-based tests have a limited but distinct place in monitoring the effect of drug treatment, mainly in a number of gastroenterological diseases.

Clinical toxicology

Drug substances like acetaminophen, methotrexate or amiodarone and also mushroom intoxication (e.g. death cap poisoning) may cause liver damage (cirrhosis), which in extreme situations may progress to acute liver failure shortly after intoxication. The extent of liver damage may be assessed by liver biopsy, biochemical tests, clinical assessments or stable isotope technology. Because of the many functions the liver has, not one test is able to draw the complete picture. Since the liver biopsy merely tests inflammation and biochemical monitoring is hardly correlated to metabolic liver capacity, the ^{13}C-breath test is considered to be the only test allowing to measure liver detoxification capacity directly. Extensive experience exists for the ^{13}C-aminopyrine breath test [106,107], by which the capacity to carry out hydroxylation and N- and O-methylation is measured. Breath tests are dynamic tests, which are able to detect liver damage in an early phase. Therefore, this test will lead to an earlier start of supportive treatment of liver damage than at the onset clinical symptoms, such as encephalopathy, when the prognosis has worsened. The use of aminopyrin as an analgesic drug has led to serious adverse events (agranulocytosis). Therefore it has been withdrawn from the market as a drug product. Since pharmacological doses are prescribed (2 mg/kg) in the breath test, aminopyrine-induced toxicity can not be excluded. Therefore this test is not preferred by most scientists. Alternatives are ^{13}C-methacetin, ^{13}C-caffein or ^{13}C-galactose breath tests.

5.7 Regulatory aspects of stable isotopes

Finished products containing stable isotopically labeled substances for medical purposes can generally not be procured. If such products are to be used in a clinical trial or in medical diagnostic procedures, the pharmacist is responsible for the product design and clinical production. Stable isotope products may be classified as food product, medicinal product or medical device. The product class determines the regulatory demands that apply for investigational or medical use. There is to the best of our knowledge no guidance document available for classifying these products. Since it is difficult to classify a stable isotope product, there is a risk of misclassification. Especially for investigators there is the risk that their product is unwillingly classified in the category "medicinal product" with a heavy regulatory burden as a consequence. In 2001 a new and more stringent set of rules was adopted by the European Union for clinical trials with medicinal products. This directive contains too strict rules for non-commercial research with drug products by increasing the administrative and

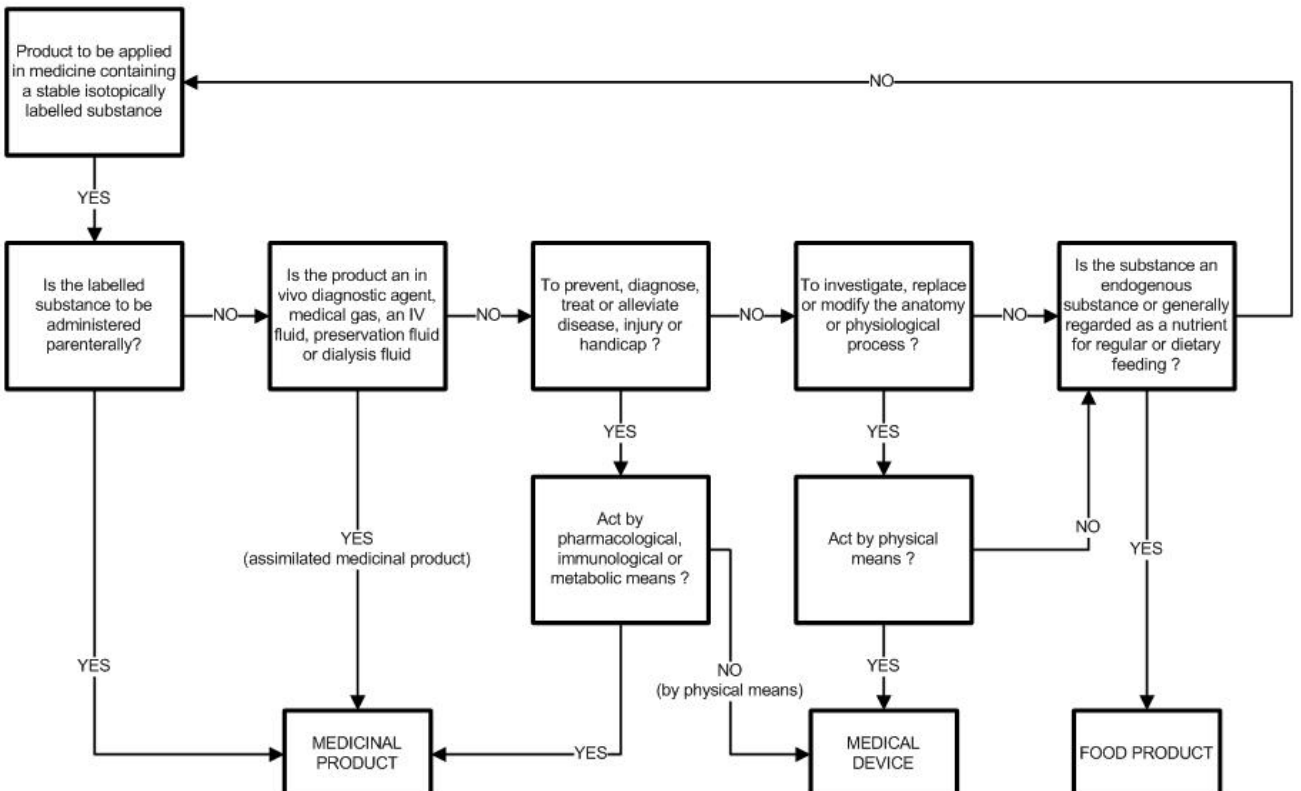


Figure 2: Decision tree to support classification of a product containing a stable isotopically labeled substance

financial burdens [108]. Based on the definitions in the EU council directives for food products [109] (2002/178/EC), medicinal products [110] (65/65/EEC) and medical devices [111] (93/42/EEC), a decision tree may be drafted to support classification of a product containing a stable isotopically labeled substance (figure 2). Based on the product class, the requirements for quality assurance can be determined. The scope and format of product design documentation as part of the clinical trial application as well as the GMP-requirements for clinical production can be determined. However, whatever product class applies, the product quality should always be assured, in order not to compromise on the quality of the trial and to protect the subject from unsafe experimentation.

A stable isotope product is classified as a medicinal product when it is administered parenterally or when applied to restore, correct or modify physiological functions by exerting a pharmacological, immunological or metabolic action or to enable a medical diagnosis [110]. Stable isotope products that are designated as medicinal products, are generally unlicensed drug products. When used in clinical investigations, they may be classified as investigational medicinal product (IMP) or non-investigational medicinal product (NIMP). IMPs are the subject of investigation in a clinical trial [112] where NIMPs perform a supportive role or are used to assess a relevant clinical trial end-point [113]. When the stable isotope product is regarded as an IMP, as such it falls within the scope of the clinical trial directive [114] (2001/20/EC). The clinical trial application needs documented product design in the form of an IMP dossier (IMPD). Clinical production may be executed by a pharmaceutical manufacturer under the provisions of an IMP manufacturer's authorization for the particular process at hand. Stable isotope products that are regarded as NIMPs are currently under discussion in the EU [113]. The clinical trial application needs documented product design but not necessarily in the form of an IMPD. On the one hand, the stable isotope product may be produced as an NIMP by a pharmaceutical manufacturer under the provisions of an IMP manufacturer's authorization for the particular process at hand. On the other hand, it may be produced under national provisions to the principles of GMP and released for use by an appropriately experienced individual [113]. For example, an NIMP can be produced as magistral or officinal preparation in a pharmacy according to Good Compounding Practices under the supervision of a pharmacist. When used in a medical diagnostic procedure, the stable isotope product is generally prepared specifically for one patient and is considered a magistral or officinal preparation [110]. It may be produced in a licensed (hospital) pharmacy under the same conditions as a NIMP.

Products that are not classified as a medicinal product but are used to investigate a physiological process, may be classified as a medical device when they act by physical means. A stable isotope product will seldom be classified as a medical device. In medical practice a stable isotope product will generally be used to perform a (medical) diagnosis. However, since it acts by being metabolized it does not fall within the scope of a medical device but within the scope of a medicinal product. In clinical trials, often a stable isotope product is ingested together with the drug

product which is being investigated. Here, the stable isotope product serves as a tool to investigate a physiological process. Nevertheless, the stable isotope product should still not be classified as a medical device since it does not achieve its principal intended action through physical means. If a drug delivery device is investigated by a marker substance labeled with a stable isotope, this combination is probably still classifies as a medical device.

Food products and nutrients cover all substances not regarded as drug substances or medical devices, i.e. not applied for a medicinal purpose or a medical indication.

We present two examples from our practice in a University Medical Center. As a first example, we present a clinical investigation into fatty acid metabolism and gall bladder function. In the study protocol stable isotope technology is used to dynamically measure bile acid kinetics. Stable isotopically labeled bile salts (e.g. 2,2,4,4-$^{2}H_{2}$-chenodeoxycholic acid) are orally administered as part of a metabolic study. Since these substances are not generally regarded as food products and act by metabolic means, they are advised to be classified as a medicinal product (NIMP).

As a second example, we present a clinical investigation into the optimal composition of an infant formula as clinical nutrition. In the study protocol stable isotope technology is applied to assess fatty acid metabolism. Stable isotopically labeled fatty acids are mixed into a conventional infant formula (e.g. 1,1,1-$^{13}C_{3}$-tripalmitin). Tripalmitin is a natural constituent of palm oil and as such considered to be a food product. Infant formula with the addition of 1,1,1-$^{13}C_{3}$-tripalmitin is therefore classified as a food product as well.

5.8 Concluding remarks and future perspectives

Stable isotope technology in clinical pharmacology is applied since 1972. It may be considered as a technology with proved and promising value, depending on the area of application in the field of clinical pharmacology. Firstly, in drug pharmacology it has obtained a distinctive place when conventional methods are not able to deliver the required information. In the near future the possibility to study the metabolism of biotechnology-derived drug substances (that may also exist endogenously) are of special interest considering the rapid developments in this field over the past years. With the advent of peptides and proteins, the study of their metabolism is important to understand and improve drug treatment. Until now, this subject has gained very limited interest. Secondly, in the assessment of drug products, stable isotope technology is well established and recognized as a method to shorten bioavailability trials and to reduce the number of subjects to be included. In the performance assessment of drug delivery systems (e.g. the release profile of modified-release drug products) stable isotope tracers are not extensively developed and investigated. Applications in this area can be further developed and improve the quality of acquired data from these studies. Only ^{13}C-urea is considered as a useful alternative to gammascintigraphy in the assessment of colon-delivery systems. Thirdly, in the

assessment of patients, stable isotope technology is able to phenotype patients and thus support personalized medicine. Since this field is currently making strong progress, it is now the time to investigate the utilization of stable isotope facilitated monitoring of metabolic processes. Geno- and phenotyping are different approaches to determine the patient's sensitivity to a drug, prior to treatment. It still has to be determined which approach the medical community should favor. We envisage a combination of geno- and phenotyping since both approaches give different but necessary and complementary information.

Translation of stable isotope technologies into commercially viable products is a dismal story. As described in this review, researchers have investigated several stable isotope tracers, but only one product (^{13}C-urea breath test) has entered the market. Failure to commercialize more stable isotope tracers is related to three factors: the unfamiliarity with the methodology due to the limited number of applications, the complex regulatory system and the small market potential. Since these roadblocks are likely not to be eliminated in the near future, application of stable isotope technologies will most probably be confined to academic centers or commercial research organizations. In specific cases, investigators, clinical pharmacologists and medical doctors will require access to this technology as a tool in clinical trials, medical diagnosis or clinical pharmacology.

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